

# Kefir-isolated bacteria and yeasts inhibit *Shigella flexneri* invasion and modulate pro-inflammatory response on intestinal epithelial cells

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## RESEARCH ARTICLE

### Abstract

The aim of this work was to evaluate the ability of a kefir-isolated microbial mixture containing three bacterial and two yeast strains (MM) to protect intestinal epithelial cells against *Shigella flexneri* invasion, as well as to analyse the effect on pro-inflammatory response elicited by this pathogen. A significant decrease in *S. flexneri* strain 72 invasion was observed on both HT-29 and Caco-2 cells pre-incubated with MM. Pre-incubation with the individual strains *Saccharomyces cerevisiae* CIDCA 8112 or *Lactococcus lactis* subsp. *lactis* CIDCA 8221 also reduced the internalisation of *S. flexneri* into HT-29 cells although in a lesser extent than MM. Interestingly, *Lactobacillus plantarum* CIDCA 83114 exerted a protective effect on the invasion of Caco-2 and HT-29 cells by *S. flexneri*. Regarding the pro-inflammatory response on HT-29 cells, *S. flexneri* infection induced a significant activation of the expression of interleukin 8 (IL-8), chemokine (C-C motif) ligand 20 (CCL20) and tumour necrosis factor alpha (TNF- $\alpha$ ) encoding genes ( $P < 0.05$ ), whereas incubation of cells with MM did not induce the expression of any of the mediators assessed. Interestingly, pre-incubation of HT-29 monolayer with MM produced an inhibition of *S. flexneri*-induced IL-8, CCL20 and TNF- $\alpha$  mRNA expression. In order to gain insight on the effect of MM (or the individual strains) on this pro-inflammatory response, a series of experiments using a HT-29-NF- $\kappa$ B-hrGFP reporter system were performed. Pre-incubation of HT-29-NF- $\kappa$ B-hrGFP cells with MM significantly dampened *Shigella*-induced activation. Our results showed that the contribution of yeast strain *Kluyveromyces marxianus* CIDCA 8154 seems to be crucial in the observed effect. In conclusion, results presented in this study demonstrate that pre-treatment with a microbial mixture containing bacteria and yeasts isolated from kefir, resulted in inhibition of *S. flexneri* internalisation into human intestinal epithelial cells, along with the inhibition of the signalling via NF- $\kappa$ B that in turn led to the attenuation of the inflammatory response.

**Keywords:** kefir microorganisms, *Shigella flexneri*, pro-inflammatory response

## 1. Introduction

Probiotics are classically defined as live microorganisms that administered in adequate amounts may confer health benefits to the host (FAO/WHO, 2002), and their use in food industry is increasing in the last decades. Numerous reports have demonstrated the ability of several species of lactobacilli, bifidobacteria and yeasts to exert beneficial

effects, including protection of a potential host against infectious diseases caused by enteric pathogens and prevention of intestinal disorders (Gupta and Garg, 2009). Generally, these probiotic microorganisms are capable of inhibiting the action of pathogens, enhancing the intestinal barrier and modulating the immune response, among other effects (Plaza-Díaz *et al.*, 2014; Servin, 2004).

Kefir is a fermented product obtained by fermentation of milk with a complex microbiota confined to 'kefir grains', in which different lactic acid bacteria and yeasts coexist in a symbiotic association. Besides, kefir consumption has been associated with several health-promoting properties (Guzel-Seydim *et al.*, 2011; Lopitz-Otsoa *et al.*, 2006); different kefir-isolated microbial strains have also shown potentiality as probiotics (Santos *et al.*, 2003; Zheng *et al.*, 2013). In this sense, the ability of several bacterial and yeast strains isolated from kefir grains to inhibit different intestinal pathogenic bacteria such as *Salmonella* spp., *Clostridium difficile*, *Shigella* spp. and *Escherichia coli* have been demonstrated by *in vitro* and *in vivo* studies performed in our workgroup (Bolla *et al.*, 2013a,b; Carasi *et al.*, 2014; Golowczyc *et al.*, 2007; Hugo *et al.*, 2008; Kakisu *et al.*, 2013; Trejo *et al.*, 2010).

*Shigella* is a Gram-negative foodborne bacillus that is one of the most frequent causes of acute diarrhoea in developing countries (Kosek *et al.*, 2010). Among different species, *S. flexneri* and *Shigella sonnei* are often isolated from children with bacillary dysentery (Merino *et al.*, 2004; Xia *et al.*, 2011). The pathogenesis of *Shigella* infection begins with an invasion of colonic and rectal epithelium followed by the intracellular bacterial replication and spread to adjacent cells (Watarai *et al.*, 1995), thus causing an intense inflammatory response that triggers a dysenteric syndrome called shigellosis (Schroeder and Hilbi, 2008). In humans, analysis of cytokine expression in rectal biopsies of infected patients at the acute phase of the disease has revealed up-regulation of pro-inflammatory genes such as those encoding interleukin (IL)-1 $\beta$ , IL-6, IL-8, tumour necrosis factor (TNF)- $\alpha$  and - $\beta$  (Phalipon and Sansonetti, 2007).

Some authors have reported that *Lactobacillus* strains are able to downregulate inflammatory responses elicited by *S. flexneri* (Tien *et al.*, 2006) or enterohaemorrhagic *E. coli* (Stöber *et al.*, 2010) on intestinal epithelial cell lines. In this context, the capability of different kefir-isolated strains of lactobacilli and yeasts belonging to CIDCA collection to modulate the flagellin-induced innate response on the Caco-2-ccl20:luc reporter system was recently reported (Carasi *et al.*, 2015; Romanin *et al.*, 2010).

We have previously reported that a microbial mixture containing three bacterial and two yeast strains isolated from kefir, inhibits the growth of *S. sonnei* *in vitro* (Bolla *et al.*, 2011). More recently, this five-strain mixture demonstrated antagonism of the invasion of Hep-2 cells by *S. flexneri* and *S. sonnei* (Kakisu *et al.*, 2013). Taking into account this background information, we aimed to evaluate the ability of this kefir-isolated microbial mixture to protect intestinal epithelial cells against *S. flexneri* invasion, as well as to analyse the effect on pro-inflammatory response elicited by this pathogen.

## 2. Materials and methods

### Microorganisms and culture conditions

The microorganisms used in this study comprised *Lactococcus lactis* subsp. *lactis* CIDCA 8221, *Lactobacillus plantarum* CIDCA 83114, *Lactobacillus kefir* CIDCA 8348, *Kluyveromyces marxianus* CIDCA 8154 and *Saccharomyces cerevisiae* CIDCA 8112, isolated from kefir grains and previously identified and characterised by Garrote *et al.* (2001), Delfederico *et al.* (2006) and Diosma *et al.* (2014). Also, a clinical isolate of *S. flexneri* strain 72, obtained from the Sor María Ludovica Interzonal Hospital (La Plata, Argentina), was used. Kefir-isolated microorganisms were cultured and propagated as described by Bolla *et al.* (2011).

### Preparation of the microbial mixture

Each microorganism was cultured individually as described above. The microbial mixture (MM) was obtained as described by Bolla *et al.* (2011). The final concentrations of viable bacteria and yeasts in MM were  $1 \times 10^9$  cfu/ml and  $1 \times 10^6$  cfu/ml, respectively. Viable counts were determined by plate counting using De Mann, Rogosa and Sharpe (MRS, Difco, Detroit, MI, USA) agar for lactobacilli, YGC (yeast extract glucose chloramphenicol agar; Biokard Diagnostic, Beauvais, France) for yeast strains, and 1.1.1. agar (Difco) for *L. lactis*. Plates were incubated at 30 °C for 24–48 h in aerobic conditions.

### Epithelial cell cultures

Caco-2, and HT-29 cells were cultured in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM; Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 15% (v/v) of foetal bovine serum (PAA Laboratories, GmbH, Pasching, Austria), 1% (v/v) non-essential amino acids (Gibco BRL Life Technologies), and antibiotics (12 IU/ml penicillin and 12 mg/ml streptomycin; Gibco BRL Life Technologies).

HT-29 cells transfected with plasmid carrying a human recombinant green fluorescent protein reporter under the control of NF- $\kappa$ B promoter (HT-29-NF- $\kappa$ B-hrGFP; Tiscornia *et al.*, 2012) were kindly provided by Dra. Mariela Bollati-Fogolín of Instituto Pasteur Montevideo. These cells were cultured in DMEM supplemented with 10% (v/v) of foetal bovine serum, 1% (v/v) non-essential amino acids and supplemented with 10% v/v GlutaMAX (Gibco BRL Life Technologies).

Cells were inoculated ( $2.5 \times 10^5$  cells per well) into 24-well tissue-culture plates (Greiner Bio One, Frickenhausen, Germany) and incubated at 37 °C (14 days for Caco-2 and 48 h for HT-29 cells) in a 5% CO<sub>2</sub>-95% air atmosphere.

## Determination of *Shigella flexneri* invasion and effect of the microbial mixture

The ability of *S. flexneri* strain 72 to invade human intestinal epithelial was assessed on Caco-2 and HT-29 cell lines. To perform control assays, monolayers were washed three times with sterile phosphate buffered saline (PBS; pH 7.2) and then  $1 \times 10^8$  cfu/ml of *S. flexneri* in 500  $\mu$ l of serum-free DMEM were added to the cell culture and incubated for 1 h at 37 °C in a 5% CO<sub>2</sub>-95% air atmosphere. The cell monolayers were washed with sterile PBS and then 1.0 ml of gentamicin (100  $\mu$ g/ml in PBS) was added to each well and incubated for 1.5 h at 37 °C in order to kill the bacteria that adhered to the surface. After incubation, the cells were washed again, and were lysed by addition of 1ml of sterile distilled water (1 h at 37 °C). Appropriate dilutions of cell lysates were plated in tryptic soy agar (Oxoid, Basingstoke, UK) and incubated for 24 h at 37 °C in order to determine the number of internalised *S. flexneri*.

For the assessment of the protective effect of MM or the individual strains, cell monolayers were pre-incubated with 500  $\mu$ l MM or individual strains ( $1 \times 10^9$  cfu/ml for bacteria and  $1 \times 10^6$  cfu/ml for yeast) for 1 h, before addition of *S. flexneri*. Some experiments in presence of DMEM supernatant previously incubated with MM for 1 h at 37 °C (MM-DMEM supernatant) were also performed.

## Modulation of innate immune response on HT-29 cells

Confluent cultures of HT-29 cells were incubated with MM or individual strains and then infected with  $1 \times 10^8$  cfu/ml of *S. flexneri* in serum-free DMEM and then incubated for 1 h at 37 °C in a 5% CO<sub>2</sub>-95% air atmosphere. After incubation, samples were homogenised in RA1 lysis buffer (GE Healthcare, Munich, Germany) to perform the extraction of total RNA. Non-infected controls and controls without microbial pre-incubation were performed. Also, cells stimulated with TNF- $\alpha$  (3 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control of activation.

## Quantification of gene expression in HT-29 cells by qRT-PCR

### RNA extraction and cDNA synthesis

Total RNA was isolated using the Illustra RNAspin Mini kit (GE Healthcare, Wauwatosa, WI USA) following the manufacturer's instructions. 100 ng of total RNA was reversed transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA).

## Quantitative PCR

Quantitative real-time PCR analyses were performed using an iCycler (Bio-Rad, Hercules, CA, USA) according to the following protocol: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of amplification with 1 min annealing/extension at 60 °C and denaturation at 95 °C for 15 s. The reaction mixture comprised Super iQ SYBR Green PCR Mix (Bio-Rad), 0.5  $\mu$ mol/l of each primer, and the respective standardised cDNA as a template. Primers for chemokine (C-C motif) ligand 20 (CCL20), IL-8, TNF- $\alpha$ , IL-6 or human actin (housekeeping gene), and relative difference calculation using the  $\Delta$ Ct method were previously described (Anderle *et al.*, 2005; Rumbo *et al.*, 2004). The results were expressed respect to the basal expression of HT-29 control cells.

## Activation of HT-29-NF- $\kappa$ B-hrGFP reporter system

Confluent cultures HT-29-NF- $\kappa$ B-hrGFP cells were incubated with MM or individual strains for 1 h at 37 °C in a 5% CO<sub>2</sub>-95% air atmosphere. The cell monolayer was washed for three times with sterile PBS, and  $1 \times 10^8$  cfu/ml of *S. flexneri* in 500  $\mu$ l of serum-free DMEM were added to the cell culture, and incubated for 1 h at the same environmental conditions. After that, 1.0 ml of gentamicine (100  $\mu$ g/ml in PBS) was added to each well and plates were incubated by 18 h at 37 °C in controlled atmosphere. Upon incubation, cells were washed three times with sterile PBS, and then harvested by trypsinisation (Gibco BRL Life Technologies). Percentage of GFP-positive cells was determined by flow cytometry using a FACSCalibur™ cell analyser (BD Bioscience, Franklin Lakes, NJ, USA). Non-infected controls and controls without microbial pre-incubation were included. Also, cells stimulated with TNF- $\alpha$  (3 ng/ml) (Sigma-Aldrich) were used as positive control of activation.

## Statistical analysis

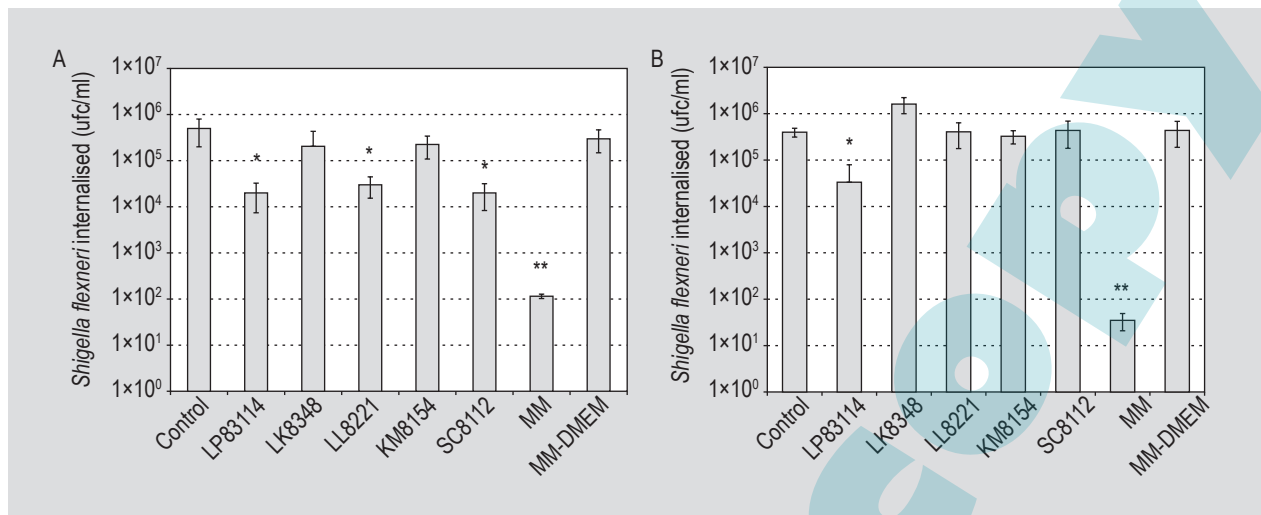
Data analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). The results were statistically tested using a Student t-test to determine any significant difference ( $P < 0.05$ ).

## 3. Results

### Effect of pre-incubation with kefir-isolated microorganisms on invasion of cultured cells by *Shigella flexneri*

In order to evaluate the effect of microbial pre-incubation on *Shigella* invasion, *in vitro* assays using two different intestinal epithelial cell lines (Caco-2 and HT-29) were performed. A significant decrease in *S. flexneri* strain 72 invasion was observed on HT-29 cells pre-incubated with MM (Figure 1A). Pre-incubation with the individual strains





**Figure 1.** Effect of microbial mixture (MM) or isolated microorganisms pre-incubation on *Shigella flexneri* 72 strain invasion in (A) HT-29 and (B) Caco-2 cultured cells. Control: *S. flexneri* infected control cells; LP83114: pre-incubation with *Lactobacillus plantarum* CIDCA 83114; LK8348: pre-incubation with *Lactobacillus kefir* CIDCA 8348; LL8221: pre-incubation with *Lactococcus lactis* CIDCA 8221; KM8154: pre-incubation with *Kluyveromyces marxianus* CIDCA 8154; SC8112: pre-incubation with *Saccharomyces cerevisiae* CIDCA 8112; MM: pre-incubation with MM; MM-DMEM: pre-incubation with MM-DMEM supernatant. Results are expressed as means  $\pm$  standard deviation. \*\*  $P < 0.05$  vs other pre-incubation treatments; \*  $P < 0.05$  vs *Shigella*-infected control cells.

*S. cerevisiae* CIDCA 8112, *L. plantarum* CIDCA 83114 or *L. lactis* subsp. *lactis* CIDCA 8221 also reduced the internalisation of *S. flexneri* into HT-29 cells although in a lesser extent than MM. No effects were observed with *L. kefir* CIDCA 8348 and *K. marxianus* CIDCA 8154 (Figure 1A).

Assays on Caco-2 cells showed that the number of *S. flexneri* strain 72 internalised after pre-incubation of enterocyte-like cells with MM significantly decreased as compared to control infected cells (around 4 log units) (Figure 1B). Interestingly, *L. plantarum* CIDCA 83114 also exerted a protective effect on the invasion of Caco-2 cells by *S. flexneri*, but the other strains under study failed to protect cells from invasion (Figure 1B). It is worth to note that pre-incubation of Caco-2 or HT-29 cells with MM-DMEM supernatant did not affect *S. flexneri* invasion on these intestinal epithelial cell lines (Figure 1).

#### Modulation of pro-inflammatory response on HT-29 cells.

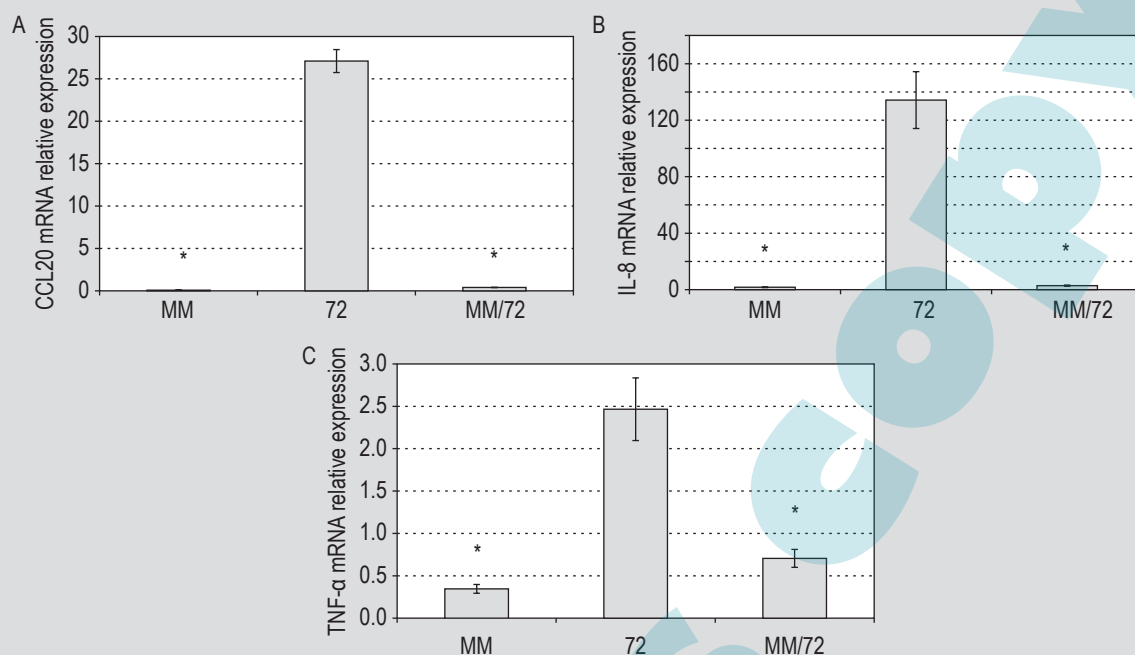
Since *S. flexneri* infection of human intestinal epithelial cells initiates an inflammatory process characterised by the induction of different cytokines and chemokines (Pédrón *et al.*, 2003), we analysed the effect of pre-incubation with MM on the expression of genes encoding TNF- $\alpha$ , IL-8 and CCL20 in *S. flexneri* infected-HT-29 cells. As it shown in Figure 2, *S. flexneri* infection induced a significant activation of the expression of *il-8*, *ccl20* and *tnf- $\alpha$*  in these cells ( $P < 0.05$ ), whereas incubation with MM did not induce the expression of any of the mediators assessed. Interestingly,

pre-incubation of HT-29 monolayer with MM produced an inhibition of *S. flexneri*-induced IL-8 (47-fold), CCL20 (68-fold) and TNF- $\alpha$  (7.8-fold) mRNA expression.

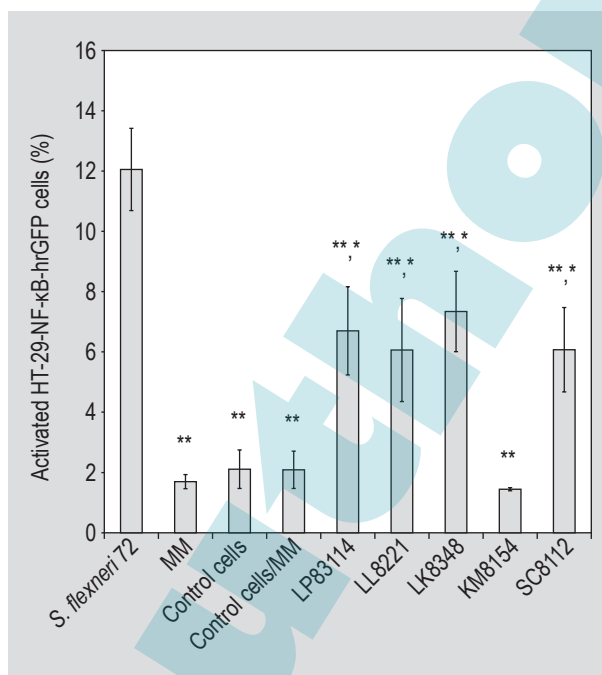
#### Modulation of *Shigella flexneri*-induced activation of HT-29-NF- $\kappa$ B-hrGFP reporter system

It has been demonstrated that gene transcription of several markers of acute inflammation induced by *S. flexneri* infection of epithelial cells, is related to the NF- $\kappa$ B pathway (Philpott *et al.*, 2000). In order to gain insight on the effect of MM or the individual strains on pro-inflammatory response of intestinal epithelial cells induced by *S. flexneri* infection, a series of experiments using HT-29-NF- $\kappa$ B-hrGFP cells were performed.

Infection with *S. flexneri* strain 72 induced a significant activation of the reporter gene (hrGFP) in the 14% of the total cells (Figure 3). This value is significantly higher than that of control unstimulated cells (around 2%). The positive control (TNF- $\alpha$  stimulated cells) showed values of 30% positive cells (data not shown). Pre-incubation of HT-29-NF- $\kappa$ B-hrGFP cells with MM dampened *Shigella*-induced activation leading to values of less than 2% positive cells (Figure 3). No activation was found in cells stimulated only with MM. Interestingly, *K. marxianus* CIDCA 8154 also interfered with the activation due to *S. flexneri*, when it was present as the sole probiotic strain. The other strains under study also inhibited activation when they were used as single strains, but in a significant lesser extent than *K. marxianus* CIDCA 8154.



**Figure 2.** Relative expression of genes encoding (A) chemokine (C-C motif) ligand 20 (CCL20), (B) interleukin 8 (IL-8) and (C) tumour necrosis factor alpha (TNF- $\alpha$ ) in HT-29 cells. 72: after infection with *Shigella flexneri* strain 72; MM/72: pre-incubation with microbial mixture (MM) and *Shigella* infection; MM: incubation with MM. Results are expressed as mean  $\pm$  standard deviation and representative of at least two independent experiments. Human  $\beta$ -actin was used to normalise gene expression. \*  $P < 0.05$  vs *Shigella*-infected control cells.



**Figure 3.** Percentage of activated HT29-NF- $\kappa$ B-hrGFP cells by *Shigella flexneri* 72 in presence or not of microbial mixture (MM) or single strains. Results are expressed as mean  $\pm$  standard deviation. \*  $P < 0.05$  vs MM pre-incubation treatment; \*\*  $P < 0.05$  vs *Shigella*-infected cells.

## 4. Discussion

In this study, we have investigated the protective effect of MM, consisting of three bacteria and two yeasts isolated from kefir, against *S. flexneri* invasion of human intestinal epithelial cells, as well as on the effect on pro-inflammatory response elicited by this bacterial pathogen. Although this five-strain mixture was shown to inhibit the growth of *S. sonnei* *in vitro* (Bolla *et al.*, 2011) as well as to antagonise the invasion of mammalian Hep-2 cells by *S. sonnei* and *S. flexneri* (Kakisu *et al.*, 2013), no studies regarding its ability to protect intestinal epithelial cells against *Shigella* infection have been previously reported.

Since the gut epithelium represents the first physical barrier against *Shigella* and other enteropathogens, the ability of probiotic microorganisms to avoid the bacterial invasion of intestinal epithelial cells, is one of the critical steps of their protective action. In this sense, several authors have shown that some strains of lactobacilli are able to inhibit the adhesion and invasion of *Shigella* on intestinal epithelial cell lines (Moorthy *et al.*, 2010; Zhang *et al.*, 2012). Our results show that the number of *S. flexneri* internalised by the intestinal epithelial cell lines Caco-2 and HT-29 significantly decreased when cells were pre-incubated with MM, which are in agreement with a similar protective effect on Hep-2 cells (Kakisu *et al.*, 2013).

However, when the effect of isolated strains was assessed, some differences were observed between both cell lines. Particularly, although *L. plantarum* CIDCA 83114 was able to antagonise *Shigella* invasion on both Caco-2 and HT-29 cells, *L. lactis* CIDCA 8221 and *S. cerevisiae* CIDCA 8112 showed only a protective effect in the model with HT-29 cells. These findings could be attributed to differences between both experimental models of human intestinal epithelium. Additionally, the inhibitory power of MM is significantly higher than that exerted by each isolated microorganism alone, suggesting a synergistic effect of MM. Similar results were reported by Moorthy *et al.* (2010) who observed that a combination of *L. rhamnosus* and *L. acidophilus* offered a better protection during *Shigella dysenteriae* infection as compared with single strains.

Taking into account that soluble metabolites could mediate the inhibition of pathogen invasion, DMEM supernatant obtained after incubation with MM for 1 h (the same time that cells were pre-incubated with kefir-microorganisms) was tested, and no effect was observed on *S. flexneri* internalisation. This suggests that the observed effects are due to direct microbe-enterocyte interaction. In this context, a competition between kefir microorganisms and *S. flexneri* for adhesion sites on intestinal epithelial cells cannot be excluded, in particular in the case of the inhibitory effect exerted by *L. plantarum*. Further research will be needed in order to test this hypothesis. On the other hand, *Shigella*-exclusion due to agglutination with yeast cells can be ruled out since no agglutination was observed in any of our previous studies with these microorganisms (Bolla *et al.*, 2011; Kakisu *et al.*, 2013).

It is known that enteroinvasive bacteria stimulate mucosal inflammation that results in severe tissue destruction. Epithelial invasion by *Shigella* and spreading to the basolateral domain lead to massive recruitment of neutrophils that contributes to the rupture of the epithelial barrier thus facilitating bacterial invasion (Pédrón *et al.*, 2003). This process is accompanied by secretion of different cytokines and chemokines by intestinal cells that in turn initiate the inflammatory process.

In the present study, the expression of IL-8, a pro-inflammatory chemokine produced by epithelial cells in response to contact between a variety of invasive microorganisms and the epithelial basolateral membrane (Eckmann *et al.*, 2000; Köhler *et al.*, 2002), was significantly increased in HT-29 infected with *S. flexneri* strain 72. An increment was also observed in these cells for the TNF- $\alpha$  encoding gene, a cytokine that plays a major role in epithelial destruction in experimental shigellosis (D'Hauteville *et al.*, 2002). The activation of expression of these two potent mediators was previously reported for HT-29 cells infected with *S. dysenteriae* (Jung *et al.*, 1995; Moorthy *et al.*, 2010) and other invasive bacteria such as *Salmonella*

Dublin, *Yersinia enterocolitica* and enteroinvasive *E. coli* (Jung *et al.*, 1995). Furthermore, infection with *S. flexneri* strain 72 induced the up-regulation of CCL20, a potent chemoattractant for dendritic cells, suggesting a potential of this strain for the recruitment of these cells which have an instrumental role in the induction of the adaptive immune response in the gut (Sierro *et al.*, 2001). In concordance with our results, other authors observed a similar activation of IL-8, CCL20 and TNF- $\alpha$  encoding genes after infection of human Caco-2 cells with an invasive strain of *S. flexneri* (Pédrón *et al.*, 2003).

Interestingly, pre-incubation of HT-29 cells with MM produced a strong inhibition of the *Shigella*-induced activation of IL-8, CCL20 and TNF- $\alpha$  gene's expression. Noteworthy, Tien *et al.* (2006) observed that *L. casei* down-regulate the transcription of a number of genes encoding pro-inflammatory effectors such as cytokines and chemokines and adherence molecules induced by invasive phenotype of *S. flexneri* in Caco-2 cells. Similarly, *L. rhamnosus* and *L. acidophilus* synergistically inhibit the pro-inflammatory response elicited by *S. dysenteriae* infection in Caco-2 cells (Moorthy *et al.*, 2010). However, the mechanism of protection and the relationship with the suppression of inflammation has not been established. According to the sequence of the events leading to the observed results, we can foresee two scenarios: the treatment with MM diminish inflammation thus leading to a decrease in *Shigella* internalisation or the diminution of *Shigella* internalisation triggers an anti-inflammatory effect. Certainly these issues deserve future research.

As far as we know, our work constitutes the first report showing the ability of a mixture of bacteria and yeasts to down-regulate the *Shigella*-induced pro-inflammatory response on intestinal epithelial cells. Our results demonstrated that both bacterial and yeast strains included in the MM are able to modify key events of the interaction between *Shigella* and eukaryotic cells. These findings are in agreement with a previous report by Romanin *et al.* (2010) who demonstrated that *S. cerevisiae* CIDCA 8112, *K. marxianus* CIDCA 8154 and other yeast strains isolated from kefir showed a high capacity to inhibit intestinal epithelial innate response triggered by different pro-inflammatory stimuli, such as flagellin, *E. coli* lipopolysaccharides or IL-1 $\beta$ .

It is known that nuclear factor (NF)- $\kappa$ B is a central regulator in the activation of numerous genes involved in pro-inflammatory responses, and diverse cell functions such as growth, differentiation, adhesion and apoptosis (Jobin and Sartor, 2000). The NF- $\kappa$ B pathway mediates the transcription of several genes related to acute inflammation, such as IL-8 and CCL20 which are activated in *S. flexneri*-infected epithelial cells (Philpott *et al.*, 2000; Tien *et al.*, 2006). By using a reporter system under the control of a

NF- $\kappa$ B promoter, we observed that either MM or isolated strains are able to modulate the *Shigella*-induced activation of the NF- $\kappa$ B pathway, suggesting that the inhibition of pro-inflammatory response is related to this signalling pathway. However, the contribution of yeast strain *K. marxianus* CIDCA 8154 seems to be crucial in the observed effect. In this sense, using several reporter systems and different stimuli, other authors have reported the inhibition of the NF- $\kappa$ B pathway by co-incubation with individual lactic acid bacteria (Stober *et al.*, 2010; Tien *et al.*, 2006) or yeasts (Romanin *et al.*, 2010; Sougioultzis *et al.*, 2006), but no results regarding mixtures of potentially probiotic microorganisms have been reported.

According to our results, although each microorganism is able to modulate NF- $\kappa$ B activation, the inhibitory power of MM is significantly higher than those of individual strains. These findings support the idea, as well as described above for the inhibition of *Shigella* internalisation, that microorganisms could act synergistically in order to protect intestinal epithelial cells from damage caused by infection.

In conclusion, results presented in this study demonstrate that pre-treatment with a microbial mixture containing bacteria and yeasts isolated from kefir, resulted in inhibition of *S. flexneri* internalisation into human intestinal epithelial cells, along with the inhibition of the signalling via NF- $\kappa$ B that in turn led to the attenuation of the inflammatory response.

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